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(71) Applicant *(for all designated States except US)*: IMPERIAL COLLEGE INNOVATIONS LIMITED [GB/GB]; 47 Prince's Gate, Exhibition Road, London SW7 2QA (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HANSEL, Trevor, Thomas [GB/GB]; 148 Broom Road, Teddington, Middlesex TW11 9PQ (GB). LECKIE, Margaret, Jean [GB/GB]; NHLI Clinical Studies Unit, Royal Brompton Hospital, Fulham Road, London SW3 6HP (GB).

(74) Agent: THOMAS, Philip; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

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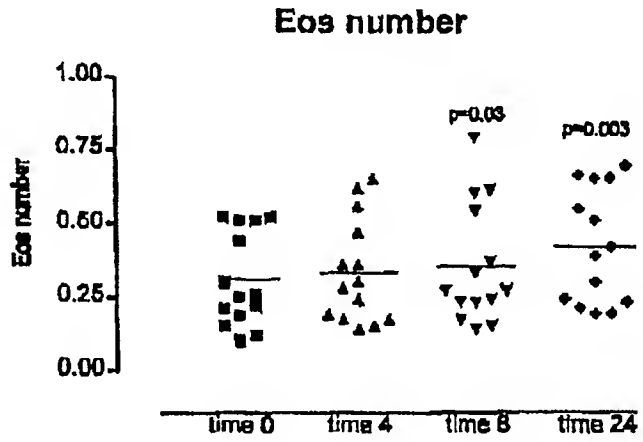

Graph 1: Eos number following allergen challenge in mild asthmatics

(57) Abstract

The invention relates to a method of measuring leucocyte activity, especially by measuring eosinophil peroxidase (EPO) and myeloperoxidase (MPO) and the use of such measurements in the diagnosis and monitoring of diseases. A preferred embodiment provides apparatus in the convenient form of a paper strip which changes colour as a result of EPO and/or MPO activity when a drop of blood is applied thereto.

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METHOD OF MEASURING ACTIVATION STATUS OF LEUCOCYTES

The present invention relates to a method of measuring the activity of an enzyme in a biological sample, especially the activity of enzymes in cells whose activity is associated with certain diseases. In particular, the invention relates to methods of measuring the activity of enzymes in leucocytes, especially peroxidase activity.

The Technicon™ H1, H2, Advia 60 and 120 haematology analysers and flow cytometers which are available from BAYER AG utilise a substrate and a chromogen (colour change agent) to stain leucocytes (white blood cells). The results of the known use of these devices are used to count and differentiate the components of whole blood, red blood cells, the various white blood cells and platelets.

The present invention seeks to provide important medical information based on measurements of the enzyme activity of selected mammalian cells. This information can be related to the presence, absence, or condition of a disease.

In a first aspect the invention provides a method of measuring the activation status of a leucocyte cell sample comprising: providing apparatus for measuring the activation status of at least one leucocyte sub-population; providing a sample containing said leucocyte; measuring the activation status of the leucocyte; comparing the measured activation status with one or more reference measurements; and relating the measured activation status of the leucocyte in the sample to the presence, absence or condition of a disease.

Preferably the activation status comprises the size and/or enzymic activity of the leucocyte. Several markers of activation of eosinophils and neutrophils can be measured colorimetrically using chemiluminescence eg. superoxide, EPO.

Preferably, the enzymic activity measurement is compared to one or more reference measurements of the same cell type and related to the presence, absence or condition of a disease.

It will be appreciated that the enzymic activity can be measured in subjects with or without a particular disease to provide a reference measurement. Hence, a comparison of the measured enzymic activity of the selected cell of the sample with the reference measurements provides an indication of the presence, absence or condition of the disease in the subject from whom or which the biological sample was taken.

Preferably the leucocyte sub-population consists of eosinophils and/or neutrophils.

Preferably the enzyme activity is peroxidase activity.

Preferably the peroxidase activity is eosinophil peroxidase (EPO) and/or myeloperoxidase (MPO).

Preferably the apparatus comprises a porous sheet material provided with at least one substrate which undergoes a colour change as a result of the enzyme activity.

Preferably the porous sheet material is provided with a lysis agent capable of lysing red cells.

Preferably the apparatus comprises an automated haematology analyser or flow cytometer.

Preferably peroxidase activity is measured by light absorption following addition of hydrogen peroxide as a substrate and a chromogen, preferably 4-chloro-1-napthol.

Preferably the activation status measurement enables a determination of the mean per cell enzymic activity per leucocyte cell type and/or the mean leucocyte cell size.

In a further aspect the invention provides a method of testing the effect of a test compound on the activation status of at least one leucocyte cell type, which activation status is associated with a disease state, comprising providing a sample of cells; treating the cells with an activator and the test compound; measuring the activation status in at least one selected leucocyte cell type; and comparing the measurement with a reference measurement of a sample which was not treated with the test compound.

The invention will now be described in more detail with reference to the accompanying drawings in which:

Figures 1a to 1f. Allergen challenge data which shows that eosinophil numbers rise after allergen challenge, EPO activity increases at 24 hours, and eosinophil size peaks at 8 hours. This has relevance when considering activation status of eosinophils as it has previously been shown

that eosinophils swell upon activation (so called "hypodense" eosinophils). There are no significant differences in neutrophils.

Figures 2a to 2d. Cross-sectional data which show that EPO activity is increased in the eosinophilic group, but there are no significant differences in the size of the eosinophils between the groups. MPO activity is decreased in the neutrophilic group, and this is reflected in the significant increase in the size of these neutrophils. The neutrophils are also larger in the COPD, severe asthmatic and eosinophilic groups.

Figures 3a to 3d. Granulocyte activation experiments (graphs 11-14) show some (but not significant) reduction in both EPO and MPO activity in the middle concentrations at 15 minutes, but a significant increase in eosinophil size.

Preferred, non-limiting, illustrative examples of the invention will now be described.

Example 1 - An automated method of measurement of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) using the Technicon™ analyser

The Technicon H1, H2, Advia 120 and 60 haematology analysers utilise a substrate and a chromogen to stain leucocytes on the basis of peroxidase activity. In accordance with the present invention additional software is applied to these results allowing accurate gating and statistical analysis of leucocyte populations.

The benefits of using this system are of an automated, well validated and calibrated machine utilising up-to-date software to obtain immediate, relatively non-invasive repeatable results. This system can be applied to

the preclinical setting (with different software), many biological fluids and most importantly has a major benefit over previous eosinophil and neutrophil protein assays in that it can be applied to patients both in the community and in the clinical setting. The method may be adapted to have utility in the community using paper strip testing of capillary blood samples using the same reagents.

(i) *Method*

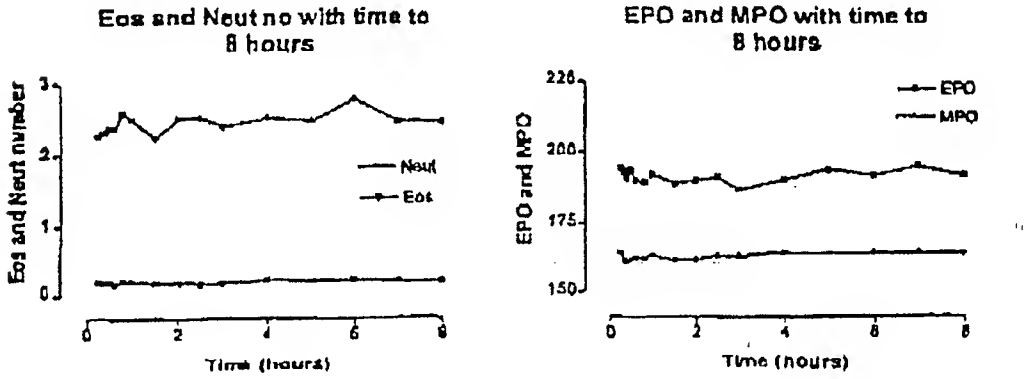
Up to 4mls of venous blood is taken into an EDTA tube and analysed within a few hours by the haematology analyser. This process takes about 90 seconds and a differential white cell count and peroxidase leucogram is generated by the method of continuous flow analysis, following the addition of hydrogen peroxide as a substrate and 4-chloro-1-naphthol as a chromogen. Cells are characterised on the basis of staining activity (light absorption) and size (light scatter), and the results are plotted on the x and y axis respectively to produce a leucocyte cytogram with the total white cell count and differential (ie. the breakdown of the population of white blood cells). This data is stored on a 5¼ inch floppy disc and later converted to DOS format (Dataget software) BAYER, Dublin, if required, onto a 3½ inch floppy. The WINMDI software program allows accurate delineation of the clusters of the eosinophils and neutrophils and enables analysis of these gated populations. Win MDI is commercially available through shareware on the internet. It is produced by TSRI Cytometry Software at bttp://facs.scripps.edu/software.html. The mean position of the cluster on the x and y axes, along with the standard deviation of these positions can be calculated using the statistical

package. The mean of the cluster position on the x axis reflects the mean per cell peroxidase activity while the y axis reflects the size of the cells.

(ii) *Preliminary Studies*

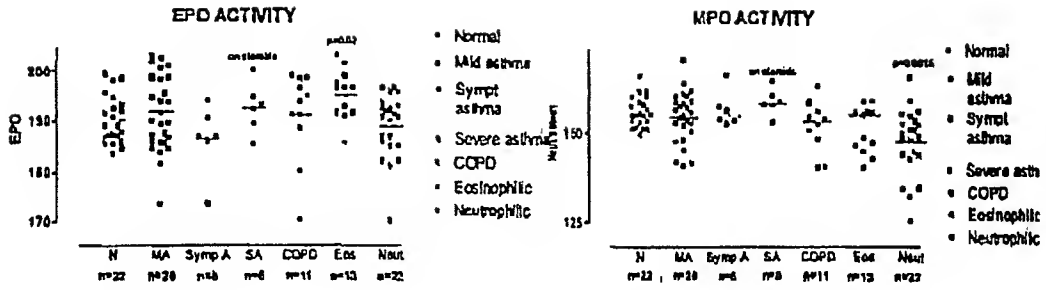
Effect of time:

Samples can be left in the EDTA tube at room temperature for up to 8 hours before distortion of the results occurs.



(iii) *Cross-sectional study:*

1. Normal subjects
2. Mild asthmatics
3. Symptomatic asthmatics on β_2 agonist only
4. Severe asthmatics
5. patients with COPD
6. Patients with eosinophilia
7. Patients with neutrophilia



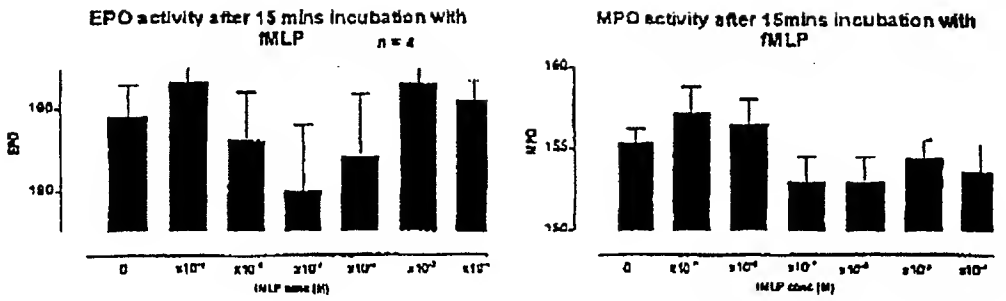
(iv) Results show that there are differences in EPO and MPO between some of these groups of subjects. In particular there is a significantly raised EPO activity in subjects with an eosinophilia, and a significantly decreased MPO activity in subjects with a neutrophilia.

(v) *In vitro granulocyte activation experiments:*

6 normal subjects.

Venous blood activated by the non-specific activator of granulocytes, formyl-methionine-leucine-proline (fMLP), in various concentrations and incubated for 15, 30, 45 and 60 minutes.

Results show that intracellular EPO levels decrease after 15 minutes incubation with formyl-methionine-leucine-proline (fMLP) at a concentration of 10^{-5}M (but not statistically significant).



(vi) *Interpretation of results:*

Samples can be left at room temperature for up to 8 hours prior to analysis. This has significance for samples taken in the community.

There are clear differences in EPO and MPO in eosinophilic and neutrophilic subjects respectively when compared to normal subjects. This may have relevance in allergic eosinophilic diseases, parasitic infestations, in autoimmune diseases and in infections causing a neutrophilia. Neutrophils have been previously shown to play a role in acute myocardial ischaemia and infarction. Further cross-sectional analysis are required in these and perhaps other disease states.

Granulocytes from whole blood can be activated *in vitro* using a stimulant. Reduction in both intracellular EPO and MPO activities are seen using the above method, and this probably reflects activation and degranulation. To clarify this, extracellular EPO and MPO can be measured at the same time-points and concentrations.

Exemplary uses of the methods of the invention

Measurement of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) in the diagnosis and monitoring of the following disease states:

A. Allergy, hayfever, asthma, food allergy.

Chronic bronchitis, chromic obstructive pulmonary disease (COPD).

B. Infections: acute appendicitis, pneumonia, urinary tract infections, post-viral fatigue (ME) syndrome, acquired immunodeficiency syndrome (AIDS), parasitic and protozoan infections, septicaemia and septic shock, immunodeficiency states.

Differentiating infection from autoimmune disease.

C. Malignancy: leukaemias, hyper-eosinophilic syndrome, myelo-proliferative disorders.

D. Drug therapy: Response to chemotherapy, drug allergy.

E. Post transplantation: bone marrow, heart, kidney.

F. Auto-immune disorders: rheumatoid arthritis, systemic lupus erythematosus (SLE), vasculitis (including Wegener's granulomatosis, Churg-Strauss syndrome).

G. Metabolic disease: liver disease, involving lack or excess of an enzyme.

H. Cardiac states: myocardial infarction, angina, during angiography eg. percutaneous transluminal coronary angiogram (PTCA), disseminated intravascular coagulation (DIC).

Other cell surface and intracellular enzyme activities on blood samples can be measured similarly to provide important medical information according to the methods of the invention. Eg:

- Surface CD enzymes: CD9
- Viral proteases: human immunodeficiency virus (HIV)
- Intracellular infections: TB
- Metabolic enzymes: lactate dehydrogenase (LDH), lysosomal enzymes
- Intra-cellular signalling enzymes

Other sources of mammalian cells include:

- Venous and capillary blood
- Sputum, bronchoalveolar lavage, nasal fluids
- Gastric, bowel and faecal derived samples
- Cells derived from tissues

Example 2 - Utilising paper strips to measure enzymic activity: application to eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activity

Asthma and allergy are inflammatory diseases: reflected in an increased number and activation state of cells that cause inflammation - blood and lung eosinophils.

In asthma and allergy, blood eosinophils are increased in number, they have low density, they release their granule proteins, they are primed to generate superoxide and leukotriene c4.

In a similar manner, bacterial infections and inflammation results in an increased number and activation state of blood and tissue neutrophils.

Derived from the measurement of cellular eosinophil peroxidase (EPO) and myeloperoxidase (MPO) in blood cells as described hereinbefore, it will be possible to measure these activities in lysed blood cells.

The activity of EPO relates to the degree of eosinophilia and allergic or asthmatic inflammation, while the activity of MPO relates to the amount of neutrophilic activity as is found in bacterial inflammation.

Community monitoring:

A paper strip that can be used by patients and physicians to measure the degree of EPO and MPO in the community. In this way, they can monitor their disease, and decide on the need for changes in their drug therapy, or see if they need to urgently seek medical advice.

A model system will be developed using paper (cellulose or other material) strips that will change colour according to the amount of EPO and MPO in a drop of blood.

The paper strip will be provided with an agent which can lyse a drop of blood (venous or a fingerprick (capillary)) or other cells (eg. sputum) applied to it. Cellular peroxidase enzymes are released and have contact with paper strip substrate reagents that are converted to coloured products.

EPO and MPO are distinct proteins with different pH optima and substrate requirements. The degree of colour intensity will inform as to the level of MPO or EPO activity in the biological sample.

Capillary blood samples (single drop of blood) will be applied to separate paper strips for MPO and EPO.

The degree (intensity) of colour will be assessed against a scaled colour chart, so that the activity of MPO or EPO can be quantified. The MPO colour relates to the number and activity of neutrophils. The EPO colour relates to the number and activity of eosinophils.

The methods and apparatus of the invention can be used for monitoring and/or diagnosis of a variety of diseases, as mentioned previously.

Example 3 - In vitro drug testing: As a screening method for large numbers of new chemical entities with potential to prevent activation of MPO and EPO

A. To add a stimulating agent (eg. formyl-methionine-leucine-proline (fMLP)) to blood and incubate, followed by measuring EPO and MPO activity at a series of time intervals.

B. Measurement of MPO and EPO at time intervals in the presence of a drug substance will measure the effect of a drug substance on activation of these enzymes.

CLAIMS

1. A method of measuring the activation status of a leucocyte cell sample comprising:

providing apparatus for measuring the activation status of at least one leucocyte sub-population;

providing a sample containing said leucocyte;

measuring the activation status of the leucocyte;

comparing the measured activation status with one or more reference measurements; and

relating the measured activation status of the leucocyte in the sample to the presence, absence or condition of a disease.

2. A method as claimed in Claim 1 wherein the activation status comprises the size and/or enzymic activity of the leucocyte.

3. A method as claimed in Claim 1 or 2 wherein the leucocyte sub-population consists of eosinophils and/or neutrophils.

4. A method as claimed in Claim 2 or 3 wherein the enzyme activity is peroxidase activity.

5. A method as claimed in Claim 4 wherein the peroxidase activity is eosinophil peroxidase (EPO) and/or myeloperoxidase (MPO).

6. A method as claimed in any one of Claims 2 to 5 wherein the apparatus comprises a porous sheet material provided with at least one substrate which undergoes a colour change as a result of the enzyme activity.

7. A method as claimed in Claim 6 wherein the porous sheet material is provided with a lysis agent capable of lysing red blood cells.

8. A method as claimed in any one of Claims 1 to 5 wherein the apparatus comprises an automated haematology analyser or flow cytometer.

9. A method as claimed in any one of Claims 2 to 5 or 8 wherein peroxidase activity is measured by light absorption following addition of hydrogen peroxide as a substrate and a chromogen, preferably 4-chloro-1-napthol.

10. A method as claimed in any one of Claims 2 to 5, 8 or 9 wherein the activation status measurement enables a determination of the mean per cell enzymic activity per leucocyte cell type and/or the mean leucocyte cell size.

11. A method of testing the effect of a test compound on the activation status of at least one leucocyte cell type, which activation status is associated with a disease state, comprising providing a sample of cells;

treating the cells with an activator and the test compound;

measuring the activation status in at least one selected leucocyte cell type; and

comparing the measurement with a reference measurement of a sample which was not treated with the test compound.

12. A method as claimed in Claim 11 wherein activation status is measured by measuring cell size and/or enzymic activity.

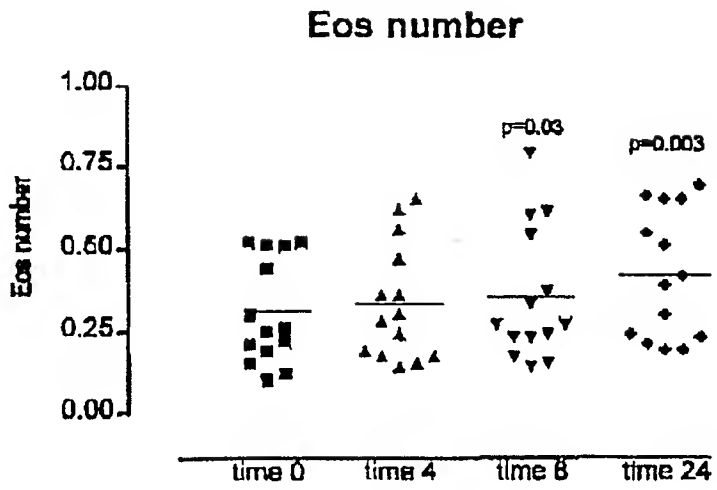
13. A method as claimed in Claim 11 or 12 wherein the selected cell type is a leucocyte sub-population, preferably eosinophils and/or neutrophils.

14. A method as claimed in any one of Claims 11 to 13 wherein the enzymic activity is peroxidase.

15. A method as claimed in any one of Claims 11 to 14 wherein the peroxidiase activity is eosinophil peroxidase (EPO) and/or myeloperoxidase (MPO).

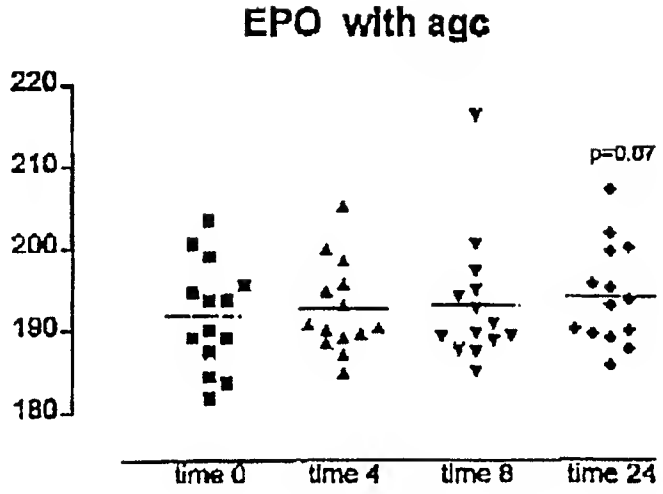
16. A method of measuring the activation status of a leucocyte in a cell sample and relating it to the presence, absence or condition of a disease state substantially as described herein with reference to one or more of the examples.

FIGURE 1A



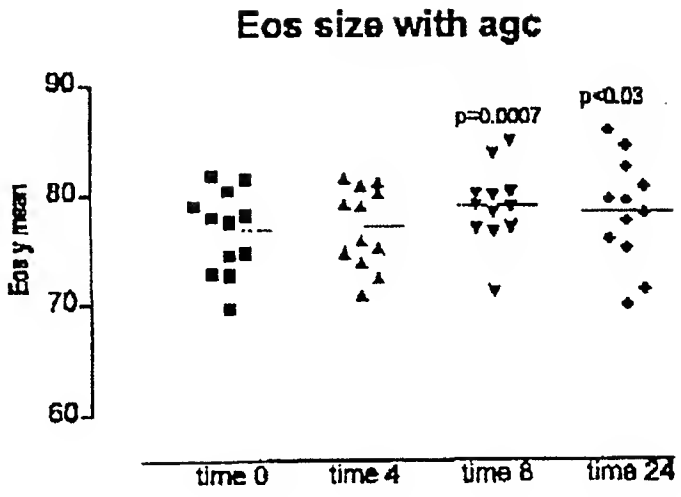
Graph 1: Eos number following allergen challenge in mild asthmatics

FIGURE 1B



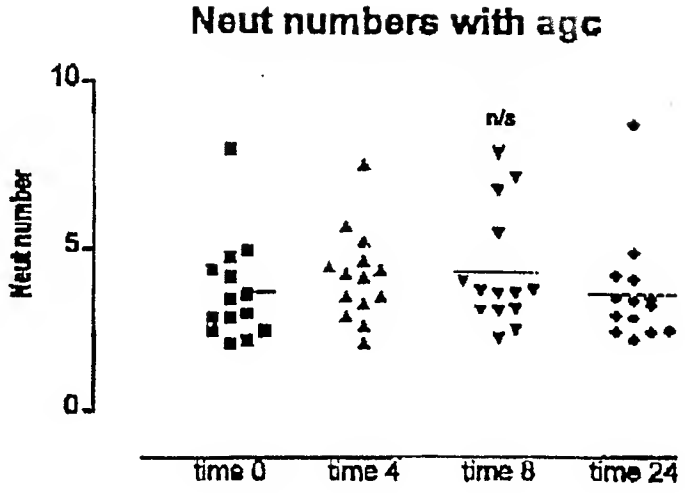
Graph 2: Eosinophil peroxidase activity following allergen challenge

FIGURE 1C



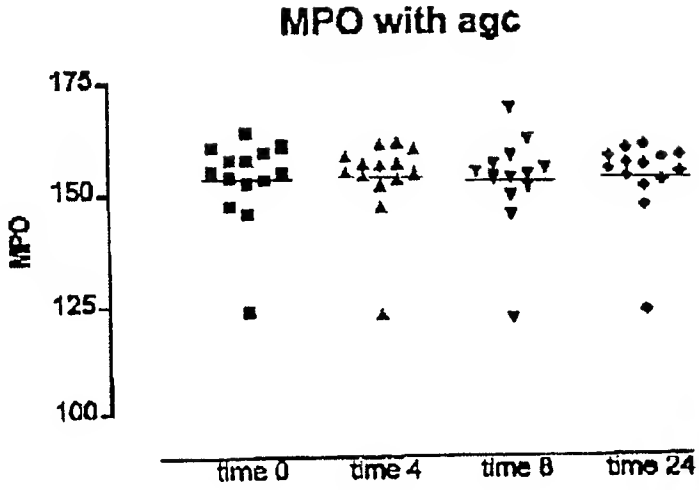
Graph 3: Eosinophil size following allergen challenge

FIGURE 1D



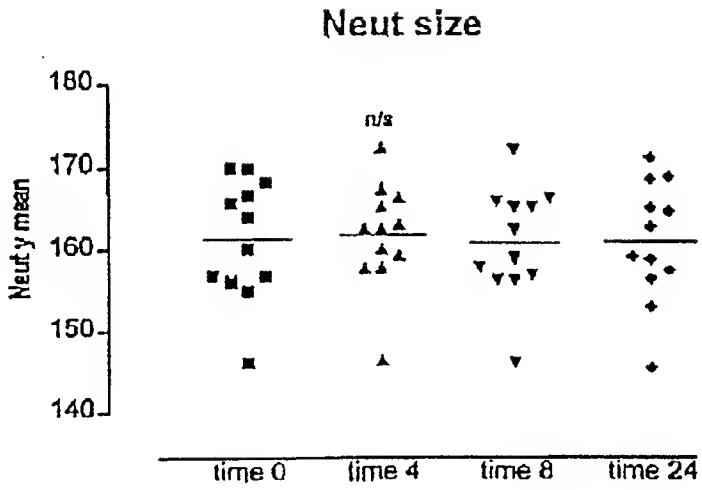
Graph 4: Neutrophil number following allergen challenge

FIGURE 1E



Graph 5: Myeloperoxidase activity following allergen challenge

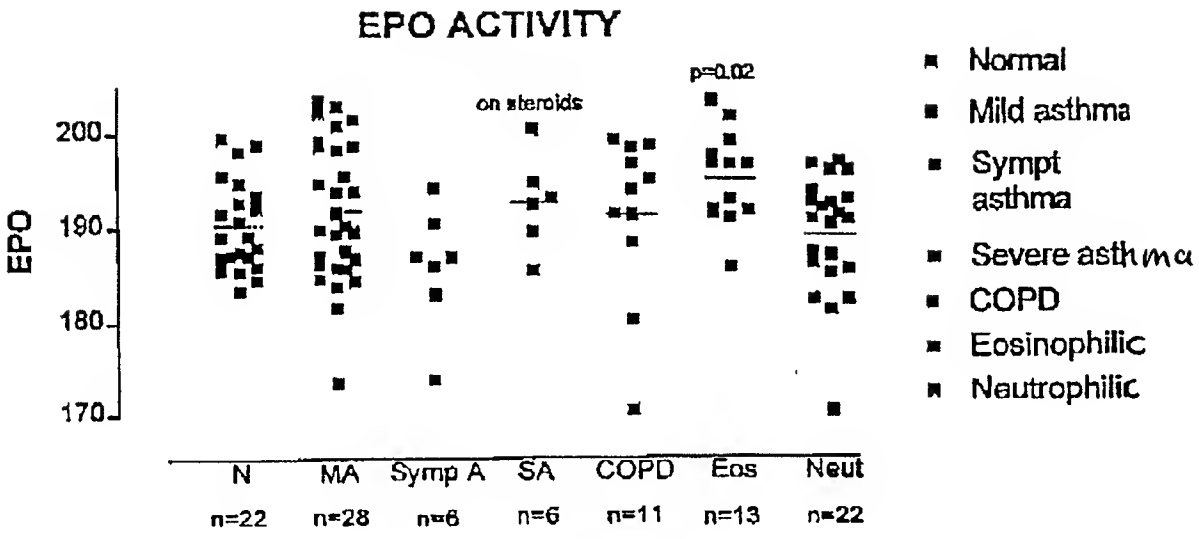
FIGURE 1F



Graph 6: NEUTROPHIL SIZE FOLLOWING ALLERGEN CHALLENGE

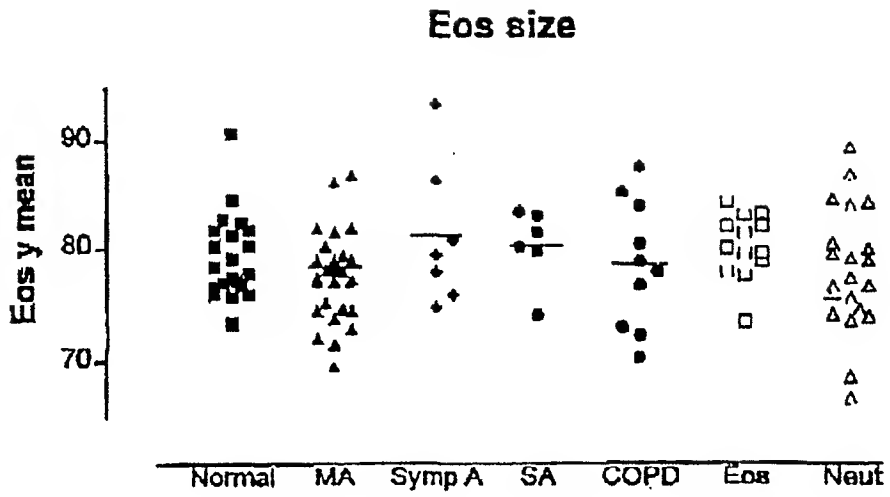
FIGURE 2A

Graph 7:



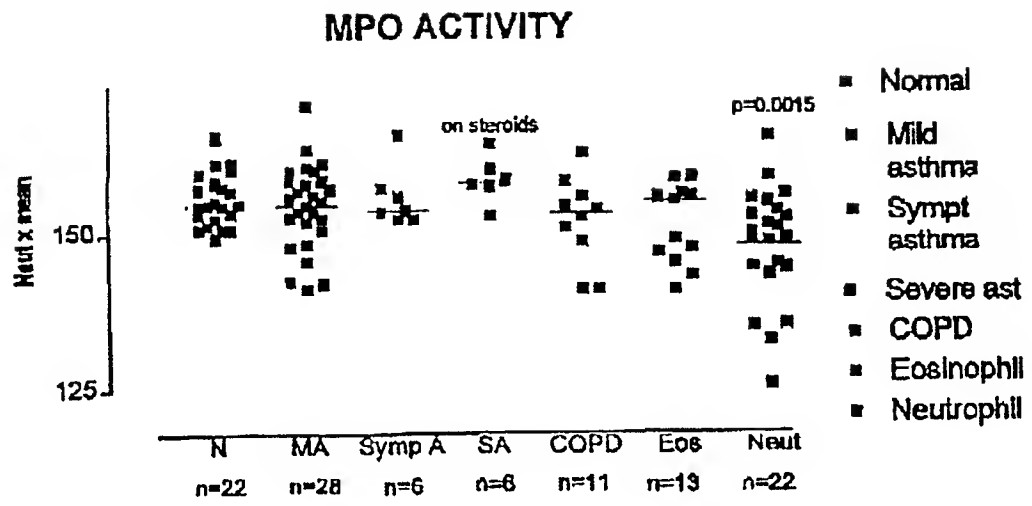
Eosinophil peroxidase activity in cross-sectional study

FIGURE 2B



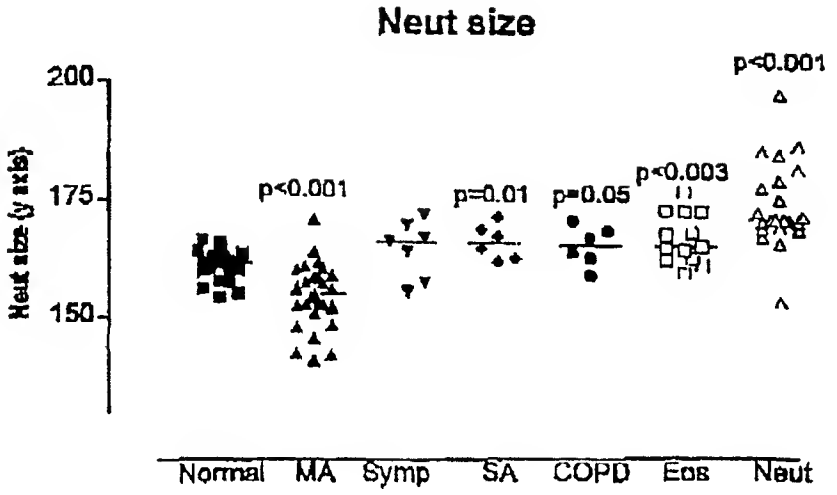
Graph 8: Eosinophil size in cross-sectional study

FIGURE 2C



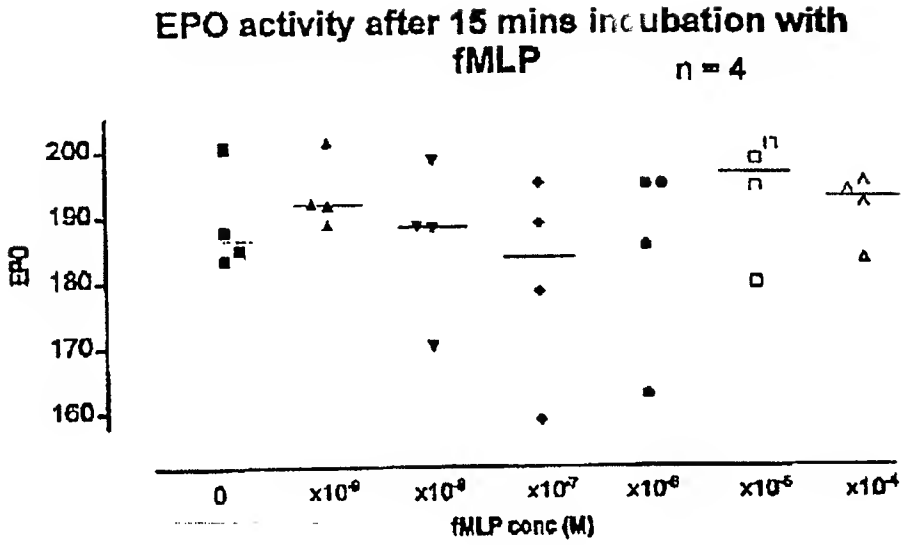
Graph 9: Myeloperoxidase activity in cross-sectional study

FIGURE 2D



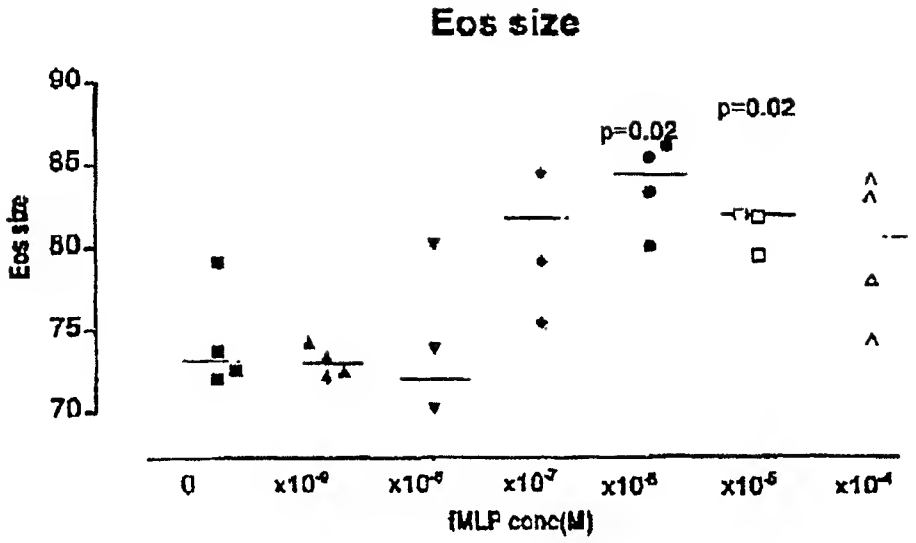
Graph 10: Neutrophil size in cross-sectional study

FIGURE 3A



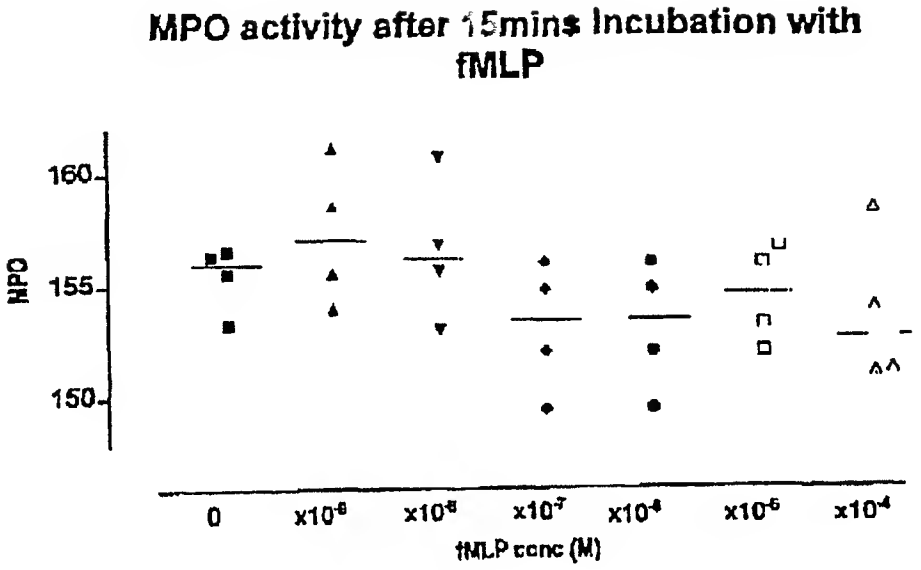
Graph 11: Eosinophil peroxidase activity following incubation with fMLP for 15 minutes

FIGURE 3B



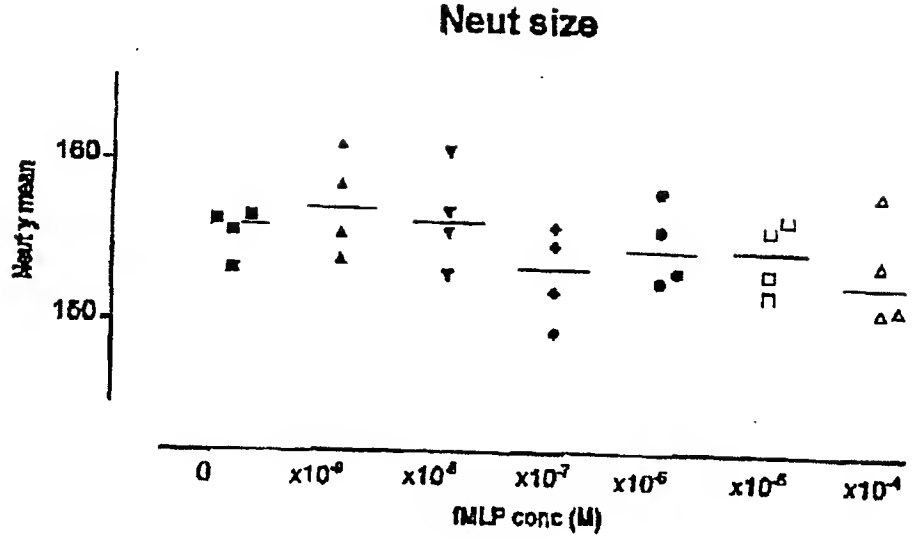
Graph 12: Eosinophil size following incubation with fMLP for 15 minutes

FIGURE 3C



Graph 13: Myeloperoxidase activity following incubation with fMLP for 15 minutes

FIGURE 3D



Graph 14: Neutrophil size following incubation with fMLP for 15 minutes